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**Loss of end-differentiated beta-cell phenotype following  
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Loss of end-differentiated beta-cell phenotype following pancreatic islet transplantation

Anderson SJ<sup>1</sup>, White MG<sup>1</sup>, Armour SL<sup>1</sup>, Maheshwari R<sup>1</sup>, Tiniakos D<sup>1,2</sup>, Muller YD<sup>3</sup>,  
Berishvili E<sup>3,4</sup>, Berney T<sup>3</sup>, Shaw JAM<sup>1</sup>

Affiliations:

1. Institute of Cellular Medicine, Newcastle University, Newcastle, UK.
2. Department of Pathology, Aretaieion Hospital, Medical School, National & Kapodistrian University of Athens, Greece.
3. Cell Isolation and Transplantation Center, Department of Surgery, Geneva University Hospitals and University of Geneva, 1211 Geneva, Switzerland;
4. Institute of Medical Research, Ilia State University, Tbilisi 0162, Georgia.

Correspondence to:

James Shaw

Professor of Regenerative Medicine for Diabetes and Honorary Consultant Physician

Diabetes Research Group, Institute of Cellular Medicine

4<sup>th</sup> Floor William Leech Building

The Medical School, Newcastle University

Framlington Place

Newcastle upon Tyne NE2 4HH

Email: [jim.shaw@ncl.ac.uk](mailto:jim.shaw@ncl.ac.uk)

## Abstract

Replacement of pancreatic  $\beta$ -cells through deceased donor islet transplantation is a proven therapy for preventing recurrent life-threatening hypoglycemia in type 1 diabetes. Although near-normal glucose levels and insulin independence can be maintained for many years following successful islet transplantation, restoration of normal functional  $\beta$ -cell mass has remained elusive. It has recently been proposed that dedifferentiation / plasticity towards other endocrine phenotypes may play an important role in stress-induced  $\beta$ -cell dysfunction in type 2 diabetes. Here we report loss of end-differentiated  $\beta$ -cell phenotype in two intraportal islet allotransplant recipients. Despite excellent graft function and sustained insulin independence, all examined insulin-positive cells had lost expression of the end-differentiation marker, urocortin-3, or appeared to co-express the  $\alpha$ -cell marker, glucagon. In contrast, no insulin<sup>+</sup> / urocortin-3<sup>-</sup> cells were seen in non-diabetic deceased donor control pancreatic islets. Loss of end-differentiated phenotype may facilitate  $\beta$ -cell survival during the stresses associated with islet isolation and culture, in addition to sustained hypoxia following engraftment. As further refinements in islet isolation and culture are made in parallel with exploration of alternative  $\beta$ -cell sources, graft sites and ultimately fully-vascularised bioengineered insulin-secreting microtissues, differentiation status immunostaining provides a novel tool to assess whether fully mature  $\beta$ -cell phenotype has been maintained.

**Introduction**

Following reproducible attainment of insulin independence through the Edmonton protocol<sup>1</sup>, islet transplantation has become an established therapy for prevention of recurrent life-threatening severe hypoglycemia in type 1 diabetes<sup>2</sup>. Although optimal islet grafts can restore non-diabetic glucose levels without adjuvant glucose-lowering therapy, detailed metabolic testing has revealed incomplete restoration of normal functional  $\beta$ -cell mass<sup>3</sup>. This is in contrast to vascularised whole pancreas transplant where normalisation of  $\beta$ -cell secretory capacity has been confirmed<sup>4</sup>. During pancreas development co-expression of insulin and the peptide hormone urocortin-3 is a phenotypic marker of full  $\beta$ -cell maturation<sup>5</sup>. Maintenance of  $\beta$ -cell urocortin-3 expression is required for physiological regulation of insulin secretion, with urocortin-3 null mice having deficient insulin secretion during periods of high glucose<sup>6</sup>. Beta-cell dysfunction, defined by loss of mature end-differentiated phenotypic markers, including urocortin-3<sup>7</sup>, and expression of non- $\beta$ -cell proteins, including glucagon<sup>8</sup>, has recently been postulated as an important contributor to impaired insulin secretion in type 2 diabetes<sup>9,10</sup>. We report abnormal  $\beta$ -cell phenotypes in two islet transplant recipients despite excellent graft function.

**Methods**

Peripheral and central tissue blocks of right and left liver lobes were fixed in buffered formalin solution and embedded in paraffin following consent from donor's family (Patient 1) and the donor (Patient 2). Pancreata optimally retrieved during organ donation after brain stem death under Research Ethics Committee approval provided non-diabetic control tissue for staining of normal adult human islets *in situ*. Four  $\mu$ m-thick tissue sections were routinely stained with haematoxylin and eosin or immunostained with antibodies for endothelial (CD31, clone JC70A, Dako, UK) or lymphatic (podoplanin D2-40, clone D240, Ventana,

USA) markers using Benchmark Ultra (Ventana, USA) automated slide staining system. Due to the limited material available for immunohistochemistry, stains were not performed on serial sections. Indirect immunofluorescence was performed in more than 15 separate intrahepatic islets in both Patient 1 and Patient 2 in addition to control pancreata using guinea pig anti-insulin (Abcam, Cambridge UK), rabbit anti-glucagon (Abcam, Cambridge UK), mouse anti-glucagon (Sigma, Gillingham, UK), and mouse anti urocortin-3 (Phoenix, Burlingame USA) antibodies. Co-expression of insulin / urocortin-3 and insulin / glucagon was confirmed by confocal microscopy Z-stack analysis.

### Case reports

Patient 1 was a 36-year-old female with type 1 diabetes of 27 years' duration who died following a cerebral hemorrhage 13 years after a single percutaneous infusion of isolated pancreatic islets pooled from two deceased donors<sup>11</sup>. Islet transplantation was undertaken for recurrent life-threatening severe hypoglycemia with poor HbA1c (11.2%)<sup>11</sup>. She attained insulin independence three months after transplant, maintaining non-diabetic oral glucose tolerance tests with HbA1c consistently <6% without any glucose lowering agents on cyclosporin, mycophenolate mofetil and low dose steroid immunosuppression.

Patient 2 was a 34-year-old female with cystic fibrosis and previous lung transplant who received a simultaneous liver and kidney transplant with islets from the same deceased donor infused into the transplant liver portal vein the following day. Induction was with thymoglobulin and ongoing immunosuppression with tacrolimus, mycophenolate and prednisolone (continued at a dose of 5 mg daily post-lung transplant without weaning). Over the next two years she required three additional islet transplants with basiliximab induction to achieve and sustain insulin-independence maintaining non-diabetic HbA1c (4.5%).

Three years after the liver transplant, she developed arterial thrombosis of the graft, leading to ischemic lesions of intrahepatic bile ducts, cholangitic abscesses and secondary hepatic failure. Despite maintained islet graft function (HbA1c 4.6%; basal C-peptide 5 ng/ml), she was treated with supplementary low dose exogenous insulin until graft hepatectomy.

**Results**

*Histological analysis of transplanted islets*

Histological analysis of liver sections from Patient 1 demonstrated engrafted islets within portal tracts (Figure 1A) with mild congestive parenchymal changes. Immunostaining for the endothelial marker CD31 confirmed that islets were situated within terminal portal vein branches (Figure 1B) or inlet venules at the portal/parenchymal interface. Further evidence that the islets were situated within vein branches was obtained through absence of immunostaining with the lymphatic marker D2-40 (Figure 1C).

Engrafted islets within the terminal portal venules were morphologically identified also in the liver explanted from Patient 2 (Figure 1D), which in addition showed evidence of ischemic and cholestatic changes in keeping with hepatic artery thrombosis, ductular cholestasis indicative of sepsis and mild steatotic changes. There was no evidence of islet fibrosis or inflammation.

*Differentiation status of transplanted islet  $\beta$ -cells*

Presence of the end-differentiated human islet  $\beta$ -cell /  $\alpha$ -cell marker, urocortin-3, was confirmed in all insulin-positive and glucagon-positive cells in normal adult islets *in situ* through immunofluorescence co-staining of five non-diabetic control pancreata

(Supplementary Figure 1). In contrast, despite co-expression of urocortin-3 in all glucagon-expressing  $\alpha$ -cells, loss of mature  $\beta$ -cell phenotype was confirmed through absent urocortin-3 staining in all insulin-positive (glucagon-negative) cells within engrafted islets in Patient 1 (Figure 2A-E) and in Patient 2 (Figure 3A-E).

In addition to loss of end-differentiation, endocrine cell plasticity was demonstrated by cells appearing to co-express insulin and glucagon in transplanted islets from both patients (Figure 2, 3 F-H) with co-localisation within single cells supported by spectral plot (Figure 3I) and Z-stack confocal microscopy (Supplementary Figure 2). Insulin / glucagon co-expressing cells were identified in all examined islets (>10 per donor).

## Discussion

We have demonstrated immunofluorescence staining phenotypes indicating loss of  $\beta$ -cell end-differentiation in islets transplanted into the portal vein despite stable engraftment and long-term maintenance of normoglycemia.

Metabolic outcomes after intrahepatic islet transplantation continue to improve and this procedure can now reproducibly deliver virtually normal overall glycemia in tandem with absolute prevention of significant hypoglycemia<sup>12</sup>. Nevertheless, complete normalisation of stimulated insulin and C-peptide secretion has remained elusive<sup>3</sup>. Loss of end-differentiated  $\beta$ -cell phenotype may provide at least partial explanation for subtle functional impairment, despite stable engraftment of sufficient  $\beta$ -cell mass to prevent hyperglycemia.

Urocortin-3 expression has been used to identify mature, fully functional,  $\beta$ -cells during pancreas development<sup>5</sup> and is expressed at approximately equal levels in  $\alpha$ - and  $\beta$ -cells in



non-diabetic adult human pancreas<sup>13</sup>. Absence of detectable beta-cell urocortin-3 staining in all transplanted islets examined in the current study provides evidence for loss of end-differentiated phenotype despite maintained insulin expression and near-normoglycemia. Loss of urocortin-3 has been reported in human  $\beta$ -cells in association with hyperglycemia in type 2 diabetes<sup>7,13</sup>. It has been proposed that urocortin-3 loss is an adaptive response to  $\beta$ -cell stress / damage, enabling the damaged  $\beta$ -cell to maintain insulin secretion without negative feedback inhibition through urocortin-3 mediated  $\delta$ -cell somatostatin secretion<sup>13</sup>. Further mechanistic studies are required to determine whether adaptation of  $\beta$ -cells to acute stress evidenced by markers of dedifferentiation including urocortin-3 loss is a direct cause of imperfect glucose control through impaired insulin secretion; or whether phenotypic changes are a reactive response to beta-cell dysfunction primarily driven by other mechanisms.

Further evidence for a phenotypic shift in intrahepatic transplanted islets was provided by identification of insulin and glucagon co-expressing cells. Each hormone appeared to be localised to different regions of the cytoplasm without incontrovertible direct overlay. In addition to confocal microscopy Z-stack imaging, spectral analysis supported the presence of bi-hormonal cells. It has been suggested that  $\beta$ -cell plasticity, leading to cells co-expressing insulin and glucagon, may contribute to abnormal glucagon secretion in type 2 diabetes<sup>9</sup>. Following islet transplantation glucagon secretion in response to hypoglycaemia remains impaired, even following replacement of near-normal pancreatic  $\beta$ -cell mass<sup>14</sup>. Whether  $\beta$ -cell plasticity, with the presence of cells expressing and secreting both insulin and glucagon, plays a role in this residual abnormality of transplanted islet function requires further study.

In the current case reports,  $\beta$ -cell pathology was not associated with decreasing functional  $\beta$ -cell mass over time or with high glucose. Amyloid deposition has been reported following

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3 intrahepatic islet transplantation, in association with failing grafts and elevated blood glucose  
4 levels<sup>15</sup>. We have previously reported near absence of amyloid deposits around islets from  
5 Patient 1<sup>16</sup>, in keeping with absence of ongoing  $\beta$ -cell stress due to maintained  
6 normoglycemia without evidence of attrition in islet function over time.  
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14 Loss of end-differentiated phenotype may facilitate  $\beta$ -cell survival during the *ex vivo* stresses  
15 associated with islet isolation and culture, in addition to sustained hypoxia following  
16 engraftment, due to peripheral as opposed to central islet revascularisation as reported by  
17 ourselves in Patient 1<sup>17</sup> and by others<sup>18</sup>. We have confirmed that islets remain in or at the  
18 periphery of the portal tracts at the portal-parenchymal interface. Additional peri- and intra-  
19 islet microvasculature may reflect neovascularisation from hepatic artery<sup>17</sup>. Alternatively, the  
20 peri-islet microvasculature could correspond to dilated collateral venules associated with  
21 portal venopathy following intraportal islet infusion.  
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34 A limitation of this study is the inability to absolutely quantify numbers of cells which have  
35 lost end-differentiated  $\beta$ -cell phenotype due to the limited tissue available within these unique  
36 samples. Nevertheless, absence of urocortin-3 staining provided a reproducible marker of  
37 altered phenotype seen in all transplanted insulin-positive / glucagon-negative cells evaluated  
38 in both recipients. In contrast, this phenotype is absent in pancreata from organ donors  
39 without diabetes, where all insulin-positive cells expressed urocortin-3. Confirmation of  
40 whether loss of urocortin-3 staining and apparent bi-hormonal cells represent a true  
41 phenotypic shift will require deeper characterisation of insulin-positive cells, comprising  
42 staining for expression and sub-cellular localisation of key  $\beta$ -cell transcription factors  
43 (including Pdx1 and Nkx6.1), examination of secretory granule ultrastructure by transmission  
44 electron microscopy and transcriptomic analysis following laser capture microdissection<sup>19</sup>.  
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Whether the phenotypic changes confirmed in the current study represent an irreversible adaptation to short term reversible peri-transplant stress; or a chronic response to ongoing  $\beta$ -cell metabolic, inflammatory or immunosuppression-induced stress despite post-transplantation near normoglycemia remains unclear. Further studies are underway to assess whether loss of mature  $\beta$ -cell phenotype reflects an adaptive response to hypoxia<sup>20</sup>.

Refinements in islet isolation and culture are ongoing in addition to innovations with the goal of fully vascularised engraftment, restoring entirely normal islet mass and function<sup>18</sup>. Evaluation of differentiation status through immunostaining co-localisation protocols provides a novel tool to assess whether fully mature  $\beta$ -cell phenotype has been maintained without dedifferentiation / plasticity following islet and isolated  $\beta$ -cell transplantation.

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### Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Figure Legends

**Figure 1. Islets engrafted within recipient liver. A, B, C Liver tissue from Patient 1. A.** Portal tract with islet within portal venule (thick arrow), interlobular bile duct (white arrowhead) and hepatic arteriole profiles (black arrowhead). Hematoxylin & eosin (H&E), x200. **B.** Islet within thin-walled portal venule (thick arrow). Endothelial vascular lining is highlighted by CD31 immunostain (thin arrows), x200. **C.** Islet within inlet venule in a septal portal tract. D2-40 immunostain decorates lymphatic endothelium (black arrowheads) but not the endothelium of portal vein (PV) or the inlet venule, confirming intravenous localisation of the engrafted islet, x200. **D. Liver tissue from patient 2.** Portal tract with islet within portal venule (thick arrow), interlobular bile duct (white arrowhead) and hepatic arteriole profiles (black arrowheads). There is ductular cholestasis (\*) and the periportal parenchyma shows hepatocellular cholestasis and mild macrovesicular steatosis, H&E x200.

**Figure 2: Changes in  $\beta$ -cell phenotype in patient 1 following intraportal islet transplantation.** Panel A demonstrates typical staining for insulin/glucagon/urocortin-3 in an islet from patient 1. Boxed region in A shows area magnified in B-E. Islet  $\beta$ -cells stained positive for insulin but all lacked urocortin-3 (UCN3) co-expression (circle indicates insulin<sup>+</sup>/UCN3<sup>-</sup> cells). All glucagon<sup>+</sup>  $\alpha$ -cells maintain UCN3 co-expression. Scale bars represent 20  $\mu$ m in A and 10  $\mu$ m in B-E. Panels F-H show islet cells (indicated by arrows) co-expressing insulin and glucagon. Scale bars represent 20  $\mu$ m in F-H. Images representative of >10 transplanted islets.

**Figure 3: Changes in  $\beta$ -cell phenotype in patient 2 following intra-portal islet transplantation.** Panel A demonstrates typical staining for insulin/glucagon/UCN3 in an islet

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3 from patient 2. Boxed region in A shows area magnified in B-E. Islet  $\beta$ -cells stained positive  
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5 for insulin but lacked UCN3 co-expression (circle indicates insulin<sup>+</sup>/UCN3<sup>-</sup> cells). Glucagon<sup>+</sup>  
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7  $\alpha$ -cells maintain UCN3 co-expression. Scale bars represent 20  $\mu$ m in A and 10  $\mu$ m in B-E.  
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10 Panels F-H show islet cells (indicated by arrows) co-expressing insulin and glucagon. Scale  
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12 bars represent 20  $\mu$ m in F-H. Images representative of >10 islets. Panel I shows a Spectral  
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14 Plot (NIS Elements AR software) obtained from the cell circled in F-H confirming insulin  
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16 and glucagon co-staining at the area indicated by the arrow in the microscopy images and line  
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18 on the graph.  
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**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Figure S1: Urocortin-3 expression in normal human pancreas.** Panel A demonstrates typical staining for insulin/glucagon/UCN3 in a representative islet within a non-diabetic deceased organ donor pancreas. All insulin<sup>+</sup> and glucagon<sup>+</sup> cells co-express UCN3. Scale bars represent 50  $\mu$ m.

**Figure S2: Z-stack confocal microscopy imaging of biohormonal cell in patient 2.** Series of Z-stack images, showing 2  $\mu$ m thick sections through an insulin / glucagon co-expressing cell. Scale bar: 10  $\mu$ m.

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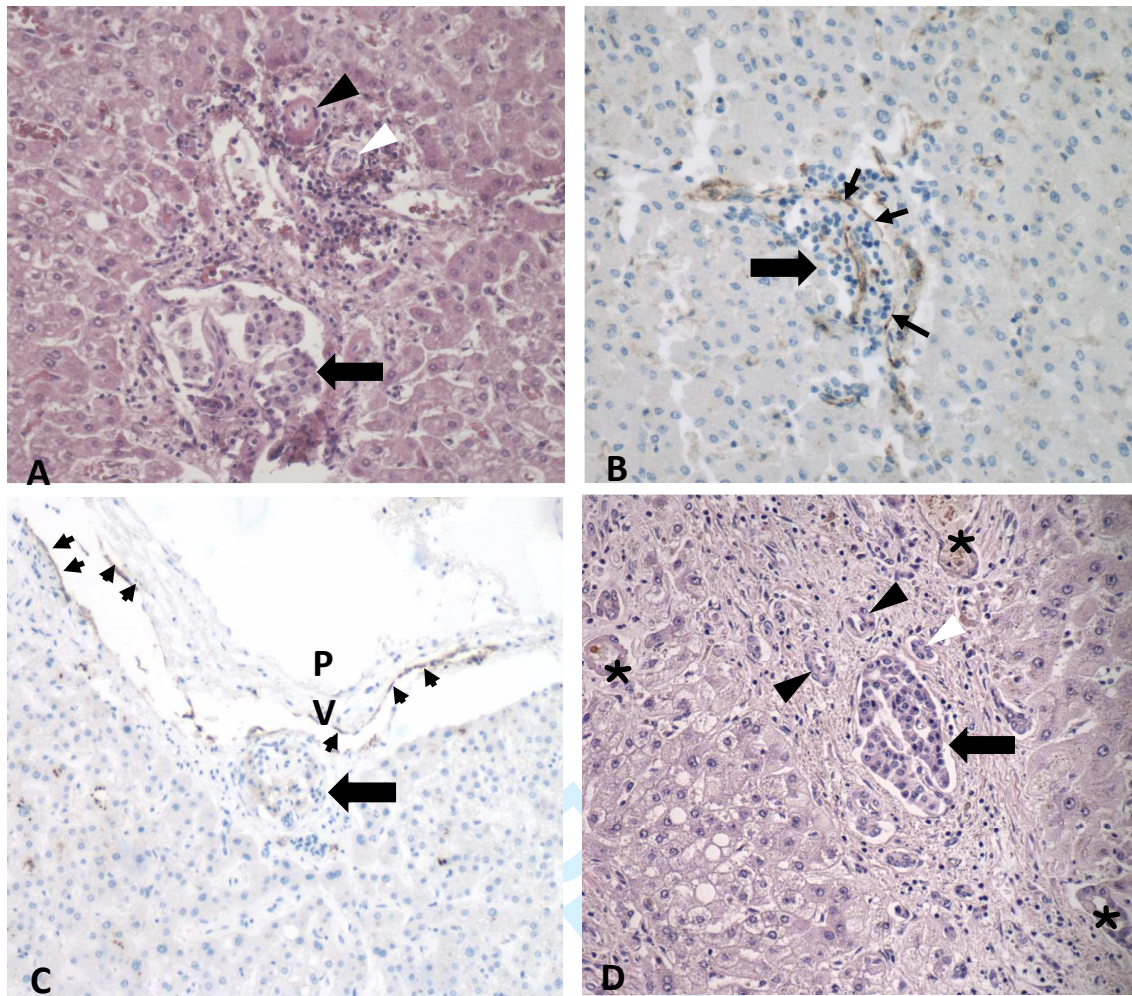


Figure 1

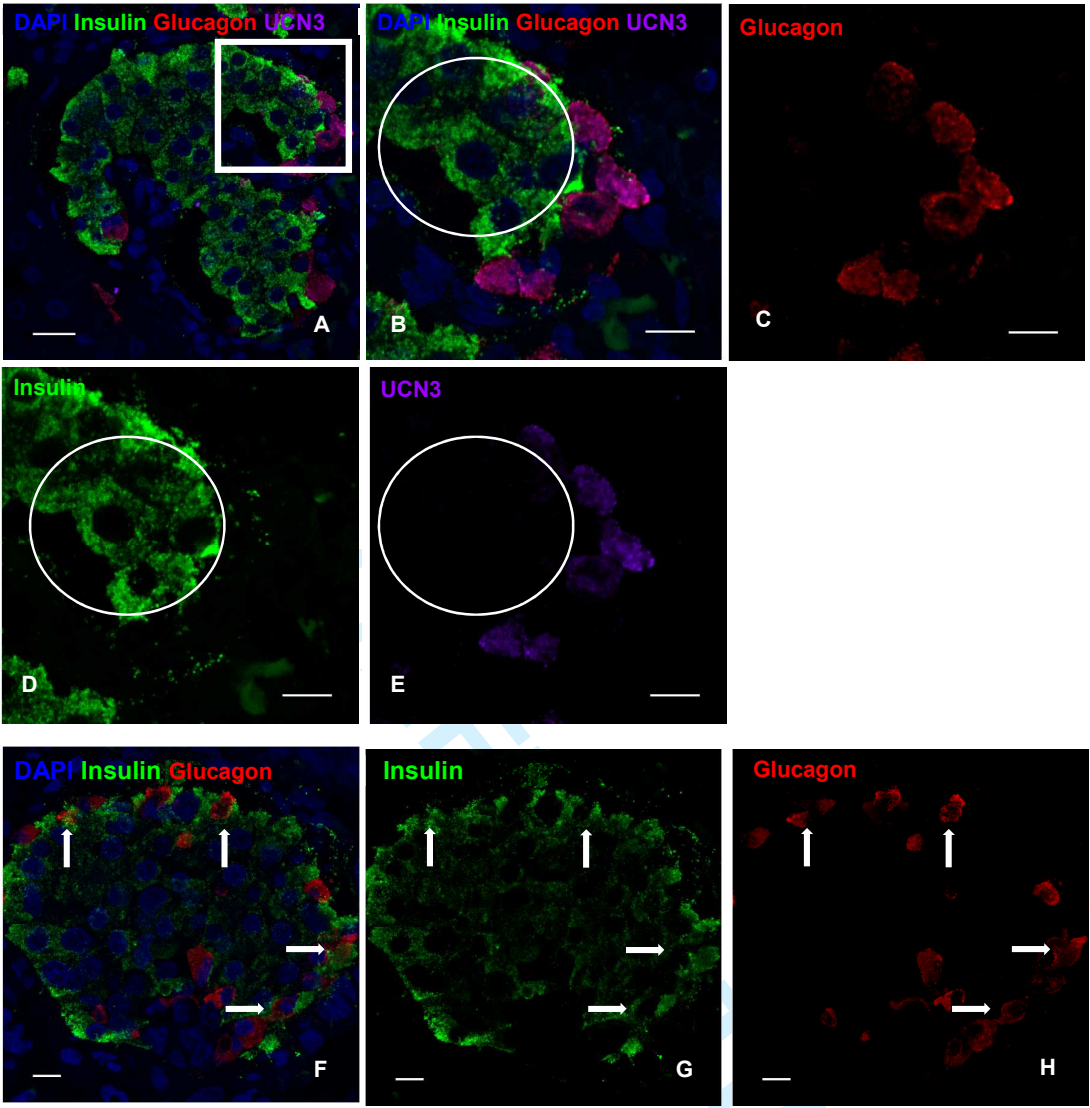


Figure 2

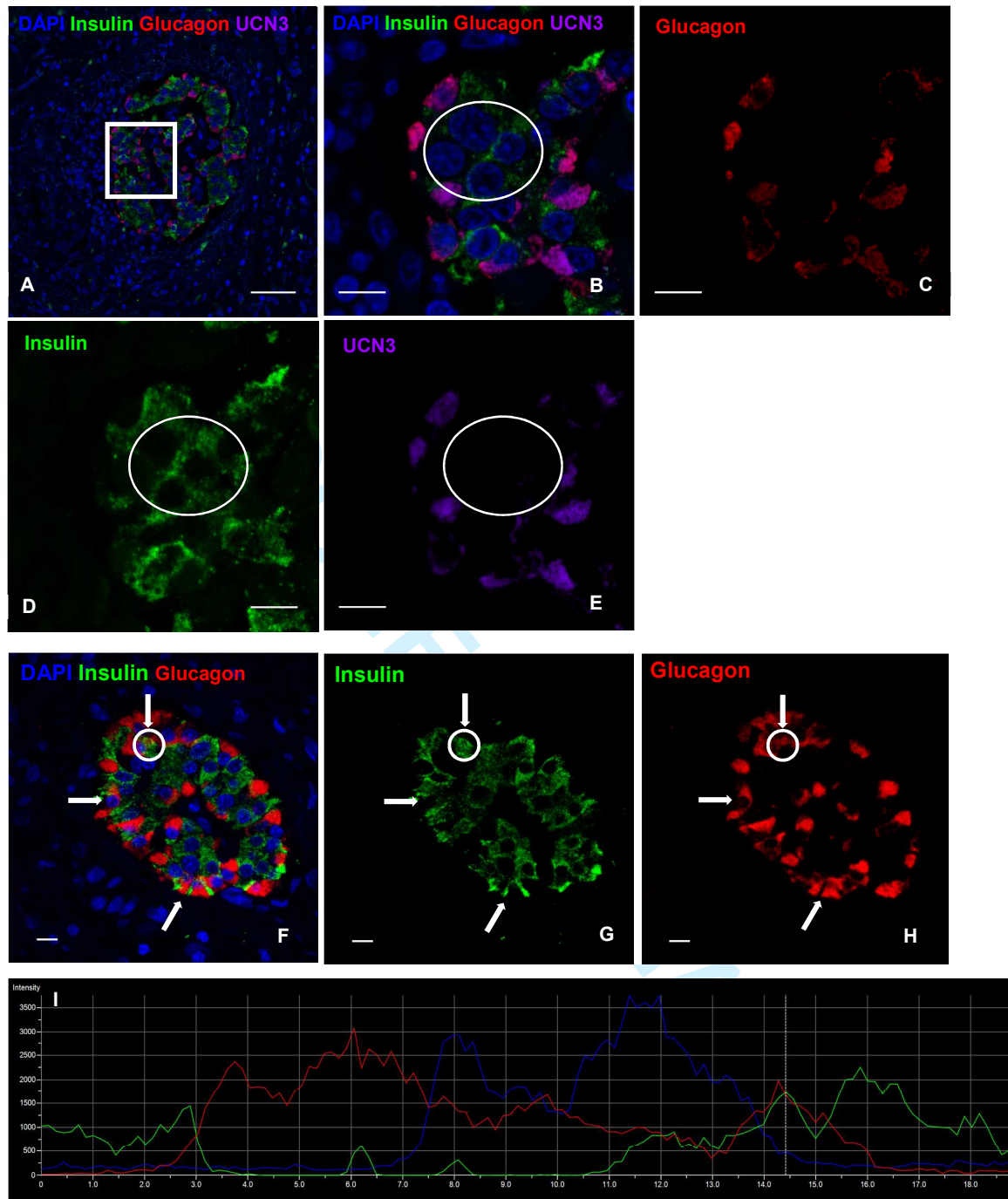


Figure 3



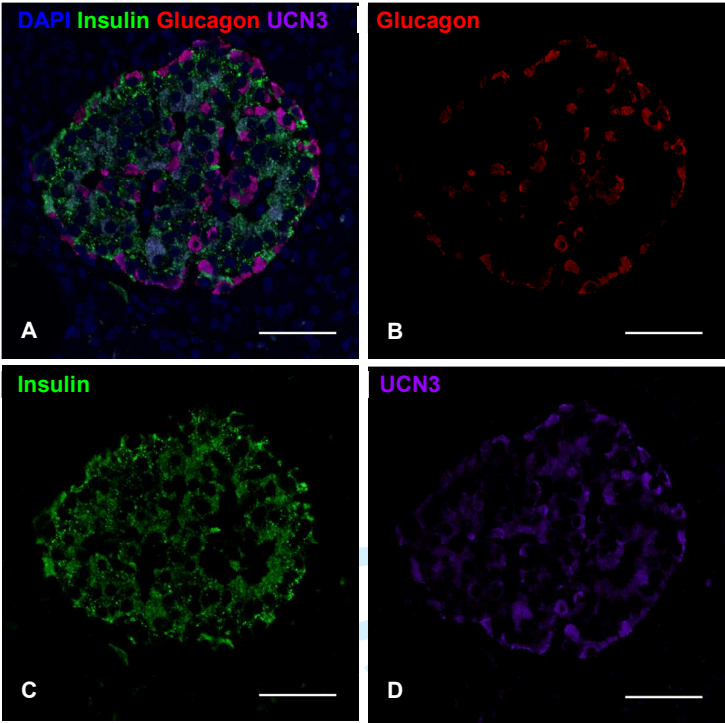


Figure S1

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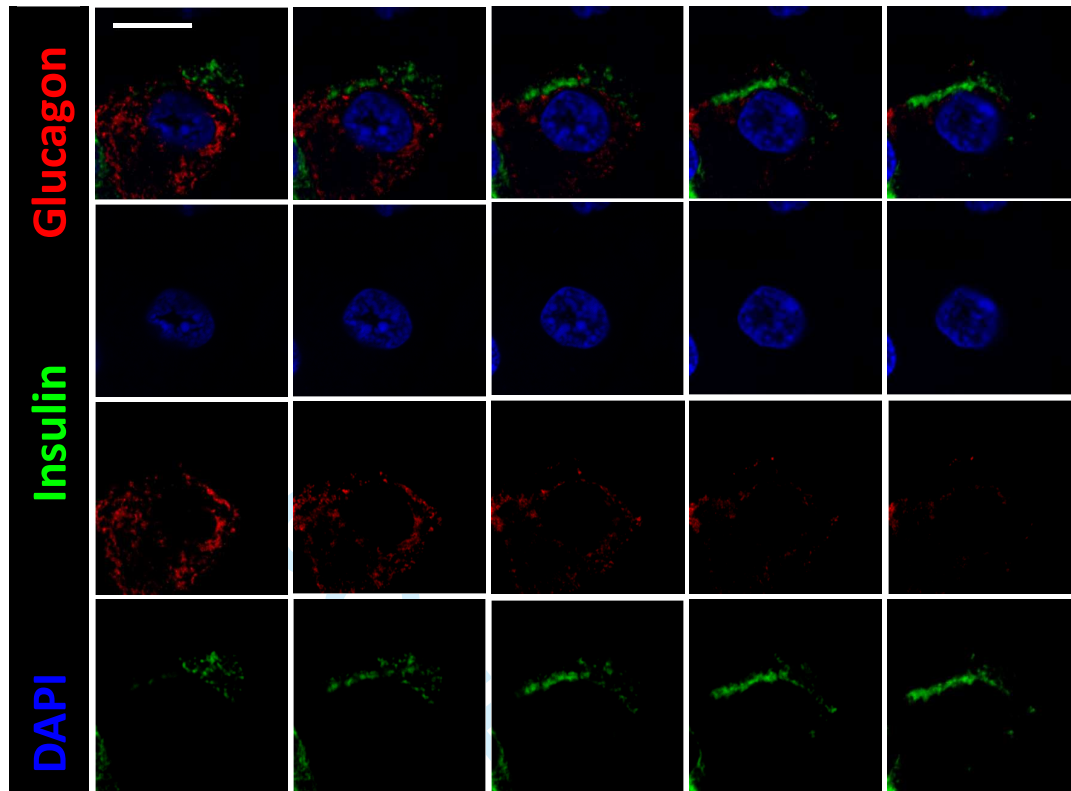


Figure S2